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Inactivation of high-risk human papillomaviruses by Holder pasteurization: implications for donor human milk banking

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27 **ABSTRACT**

28

29 AIMS: Several studies have recently reported the detection of oncogenic human papillomaviruses
30 (HPV) in human milk of a minority of lactating mothers. These findings raised safety concerns in
31 the context of human donor milk banking given the potential risk of HPV transmission to recipient
32 infants. The aim of this study was to investigate whether the Holder pasteurization, a procedure
33 currently in use in human donor milk banks for milk pasteurization, completely inactivate high-risk
34 and low risk HPV. METHODS: HPV pseudoviruses (PsV) were generated, spiked into cell culture
35 medium or donor human milk and subjected to thermal inactivation. HPV PsV infectivity and
36 morphological integrity was analyzed by cell-based assay and by electron microscopy respectively.
37 RESULTS: The Holder pasteurization completely inactivated the infectivity of high-risk (types 16
38 and 18) and low-risk (type 6) HPV both in cell culture medium and in human milk causing PsV
39 particles disassembly. CONCLUSIONS: The results presented here indicate that the Holder
40 pasteurization is an efficient procedure to inactivate high-risk and low-risk HPV thus preventing the
41 potential risk of their transmission through human donor milk.

42

43 INTRODUCTION

44 Human papillomaviruses (HPV) are non-enveloped, double-stranded DNA viruses belonging to the
45 *Papillomaviridae* family (15). HPV are classified into low-risk and high-risk groups according to
46 their oncogenic potential. Low-risk HPV types, such as HPV-6 and HPV-11, can cause benign
47 lesions of the anogenital areas, oral papillomas, conjunctival papillomas, as well as low-grade
48 squamous intra-epithelial lesions of the cervix. Perinatally acquired low-risk HPV can also cause
49 recurrent respiratory papillomatosis in infants and young children (19). Infection with high-risk
50 HPV types (primarily types 16, 18, 31, and 45) can cause cervical cell abnormalities that are
51 precursors to cancer (21). HPV types 16 and 18 cause about 70% of all cases of invasive cervical
52 cancer worldwide, with type 16 having the greatest oncogenic potential (5). Moreover, they are also
53 implicated in some head and neck cancers. The sexual activity is considered the primary route of
54 transmission of high-risk HPV among adult individuals (18), however non-sexual routes are
55 currently under investigation such as the vertical transmission through maternal milk. Indeed, in
56 2008 a paper by Sarkola et al. (28) reported that 10 out of 223 breast-milk samples (4.5%) collected
57 from Finnish women tested positive for HPV-16 raising the concern that high-risk HPV infections
58 might be transmitted to infants via breast feeding. Recent studies by Cazzaniga et al., in Italy (9),
59 Yoshida et al., in Japan (33) and Glenn et al., in Australia (13) confirmed the presence of high-risk
60 HPV sequences in the milk of a minority of lactating women. By contrast, Mammas et al., could not
61 detect any HPV sequences in milk samples from Greek women (22). To date, the possibility that
62 HPV may be transmitted to infants through breast feeding causing clinically relevant infections
63 remains to be investigated.

64 Beside breast feeding, another context in which the potential risk of HPV transmission to recipient
65 infants should be taken in to account is that of human donor milk banks. Donor breastmilk from
66 milk banks is known to contribute to the survival and well being of premature and sick babies,
67 particularly when born extremely premature and with very low birth weight. Moreover, it has been
68 shown that where new milk banks are established, the provision of donor milk can increase the rates

69 of breastfeeding on discharge from hospital of these babies (2). In this context the microbiological
70 safety of donor milk relies mainly on donor selection and on milk pasteurization with the Holder
71 method (62.5°C for 30 min) (17, 3, 1). Indeed, the Holder procedure has been shown to completely
72 inactivate some viral pathogens that can cause clinically relevant infections to the infants when
73 transmitted by maternal milk. These include the human immunodeficiency virus (HIV), the human
74 T-lymphotrophic virus type I and II (HTLV-I and HTLV-II) and the human cytomegalovirus
75 (HCMV) (12, 25, 31, 11, 14). By contrast, despite the concerns raised by the detection of oncogenic
76 HPV in maternal milk, there are no data demonstrating the safety of Holder pasteurization for the
77 inactivation of HPV. The difficulties to study HPV infectivity are due to the fact that these viruses
78 cannot be cultured using conventional monolayer cell lines as they replicate exclusively in stratified
79 squamous epithelial tissues (15). To overcome this obstacle, John Schiller and coworkers developed
80 systems to efficiently produce high-titer HPV-based gene delivery vectors, known as HPV
81 pseudoviruses (PsV) in cultured cell lines (6, 7). HPV PsV, are made of the two recombinant capsid
82 protein L1 and L2 which assemble into particles that are indistinguishable from authentic HPV
83 capsids and are capable of efficiently delivering reporter plasmids to a wide variety of cell lines. For
84 this reason HPV PsV have rapidly become a useful tool for studying HPV infectivity (8, 10, 20).
85 The aim of this study was to exploit the HPV PsV-based assays and electron microscopy to
86 investigate the effects of Holder pasteurization on high-risk and low risk HPV. The data obtained
87 demonstrate that Holder pasteurization completely inactivates HPV infectivity in cell culture
88 medium and in human milk.

89

90 MATERIALS AND METHODS

91 **Milk samples.** Breast milk samples were collected from healthy volunteer mothers with normal
92 Papanicolaou smear tests. Informed consent for breast milk donation was obtained from lactating
93 mothers of newborns admitted in the Neonatal Care Unit of University of Torino. This study was
94 approved by the Ethical Committee of the Italian Association of Human Milk Banks.

95 **Cells.** The 293TT cell line, derived from human embryonic kidney cells transformed with the
96 simian virus 40 (SV40) large T antigen, was cultured in Dulbecco's modified Eagle's medium
97 (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10 % fetal calf
98 serum (FCS; Gibco- BRL), Glutamax-I 1 % (Invitrogen, Carlsbad, CA) and nonessential amino
99 acids 1 % (Sigma Aldrich, Steinheim, Germany). 293TT cells allow high levels of protein to be
100 expressed from vectors containing the SV40 origin due to overreplication of the expression plasmid
101 (Buck et al. 2004). Low-passage-number human embryonic lung fibroblasts (HELFs) were grown
102 as monolayers in minimum essential medium (MEM) (SIGMA) supplemented with 10% fetal calf
103 serum (FCS) (SIGMA), 1 mM sodium pyruvate and 1% antibiotic-antimycotic solution (Zell
104 Shield, Minerva Biolabs GmbH, Berlin, Germany).

105 **Virus.** A (BAC)-derived HCMV strain Towne incorporating the GFP sequence (23) was
106 propagated on HELFs. The GFP marker facilitates identification of infected eukaryotic cells. Viral
107 stocks were prepared by infecting HELFs at a virus-to-cell ratio of 0.01. The cells were incubated in
108 MEM supplemented with 2% heat-inactivated FCS and cultured until a marked cytopathic effect
109 was observed. Stocks were centrifugally clarified and frozen at -80°C . Virus titers were determined
110 by plaque assay on HELFs.

111 **HPV PsV production.** Plasmids and 293TT cells used for pseudovirus (PsV) production were
112 kindly provided by John Schiller (National Cancer Institute, Bethesda, MD). HPV-16, HPV-18 and
113 HPV-6 PsVs were produced according to previously described methods (6). Briefly, 293TT cells
114 were transfected with plasmids expressing the papillomavirus major and minor capsid proteins (L1
115 and L2, respectively), together with a reporter plasmid expressing the secreted alkaline phosphatase

116 (SEAP), or green fluorescent protein (GFP). Capsids were allowed to mature overnight in cell
117 lysate; the clarified supernatant was then loaded on top of a density gradient of 27 to 33 to 39%
118 Optiprep (Sigma-Aldrich, St. Louis, MO) at room temperature for 4 h. The material was centrifuged
119 at 234,000 X g for 3.30 h at 16 °C in an SW50.1 rotor (Beckman Coulter, Inc., Fullerton, CA) and
120 then collected by bottom puncture of the tubes. Fractions were inspected for purity in 10% sodium
121 dodecyl sulfate (SDS)–Tris–glycine gels, titrated on 293TT cells to test for infectivity by SEAP or
122 GFP detection, and then pooled and frozen at -80 °C until needed. The L1 protein content of PsV
123 stocks was determined by comparison with bovine serum albumin standards in Coomassie-stained
124 SDS-polyacrylamide gels.

125 **HPV inactivation assays.** For the SEAP-based assays, 293TT cells were preplated 24 h in advance
126 in 96-well tissue culture-treated flat bottom plates at a density of 20000 cells/well in 100 µL of
127 neutralization buffer (DMEM without phenol red, 10 % heat-inactivated fetal bovine serum, 1 %
128 glutamate, 1 % nonessential aminoacids, 1 % antibiotic-antimycotic solution, and 10 mM Hepes).
129 Diluted PsV stocks equivalent to a final concentration of approximately 1 ng/ml L1 or increased
130 volumes of PsVs (equivalent to approximately 2 and 4 ng/ml L1) were incubated for 30 minutes in
131 ice (4°C), at room temperature (25°C) or at increasing temperatures in a PCR thermocycler (37°C,
132 45°C, 50°C, 55°C or 62.5°C) and then cooled at 4°C. The PsV solution (100 µL/well) was
133 transferred onto the preplated cells and incubated for 72 h. After incubation, 25 µL of supernatant
134 was harvested. The SEAP content in the supernatant was determined using a Great Escape SEAP
135 Chemiluminescence Kit (BD Clon- tech, Mountain View, CA) as directed by the manufacturer.
136 Thirty minutes after the addition of the substrate, samples were read using a Wallac 1420 Victor
137 luminometer (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA). The SEAP activity
138 is expressed as counts per second (CPS).

139 For the GFP-based assays diluted PsV stocks were incubated for 30 minutes at 4°C or 62.5°C and
140 then cooled at 4°C. The final concentration of PsV was approximately 1 ng/ml L1. The PsV

141 solution was transferred onto the preplated cells and incubated at 37°C. After 72 h the GFP-
142 expressing infected cells were observed on an inverted Zeiss LSM510 fluorescence microscope.

143 **HCMV inhibition assays.** HELF cells were seeded into 96-well plates at a density of 10⁴
144 cells/well, and incubated at 37°C in a 5% CO₂ atmosphere for 24 h. Cells were infected with GFP-
145 expressing HCMV, preincubated for 30 minutes at 4°C in ice or at 62.5°C in a PCR thermocycler,
146 at a multiplicity of infection (MOI) of 1 for 2 hours at 37°C in MEM supplemented with 2% of
147 heat-inactivated FCS. Then monolayers were washed three times and medium supplemented with
148 10% FCS was replaced. Three days postinfection, cells infected were examined by an inverted
149 Zeiss LSM510 fluorescence microscopy.

150 **Virus spiking experiments.** Raw milk samples from mothers were spiked with an inoculum of
151 HPV-16 Psv (GFP) preparation (40 ng/ml L1) or cell-free culture supernatant of HCMV (GFP) (10⁴
152 pfu per milliliter raw milk) for Holder pasteurization experiments.

153 **Holder pasteurization.** Each 100 µL of spiked milk specimens were incubated for 30 minutes at
154 62.5°C in a water bath, a procedure currently in use in human donor milk banks for milk
155 pasteurization, and then cooled at 4°C. An additional sample of spiked milk was incubated for 30
156 min at 4°C as a control. The virus-milk mixtures were then diluted in cell culture medium (1:2) and
157 added to preplated cells to test virus infectivity, as previously described.

158 **Electron microscopy.** An aliquot of diluted HPV-PsV preparation was allowed to adsorb for about
159 3 min on carbon and formvar-coated grids and then rinsed several times with water. Grids were
160 negatively stained with 0.5% uranyl acetate and excess fluid was removed with filter paper.
161 Observations and photographs were made using a CM 10 electron microscope (Philips, Eindhoven,
162 The Netherlands).

163 **Data analysis.** Each data is the mean ± standard deviation of the mean for three determinations
164 performed in duplicate. For the SEAP-based PsV neutralization assays the mean value obtained at
165 different temperatures was expressed as a percentage of the mean value obtained at 4°C. Statistical
166 analysis was performed by analysis of variance (ANOVA) followed by a Bonferroni test if *P* values

167 showed significantly differences (P value < 0.05), using GraphPad Prism 5.00 (GraphPad
168 Software).

169

170 **RESULTS**

171 **Characterization of purified HPV-16 PsV.** For this study HPV-16 was chosen as a pivotal model
172 because it is the genotype identified most often in human milk samples (28, 9, 33, 13). In
173 preliminary experiments, the quality of the HPV-16 PsV preparations was assessed by SDS-PAGE
174 and electron microscopy analysis. As shown in Fig. 1A, a major band migrating at 55 kDa was
175 detected by SDS-PAGE followed by Coomassie brilliant blue staining (lane 1). The major band was
176 confirmed to be the L1 major capsid protein by Western blot analysis with anti-L1 antibody (lane
177 2). No L1-reactive proteolytic degradation products were observed at molecular masses below 55
178 kDa demonstrating the quality of the preparation. Figure 1B shows an electron micrograph of the
179 same PsV stock. The PsV particles exhibited an average diameter of 50 to 60 nm, similar to that of
180 an authentic HPV capsid, and appeared as individual, well-assembled, icosahedral capsids. Similar
181 results were obtained with the other HPV PsV types used in this study (data not shown).

182 **Effect of heat treatment on HPV-16 PsV infectivity in cell culture medium.** To assess the effect
183 of heat treatment on HPV infectivity, HPV-16 PsV diluted in cell culture medium, were exposed to
184 increasing temperatures ranging from 4°C to 62.5°C for 30 minutes and then used to infect 293TT
185 cell cultures. Viral infectivity was quantitatively assessed by measuring the SEAP levels in the cell
186 culture supernatants. As shown in Fig. 2, PsV were stable at 4°C and 25°C, whereas exposure to
187 45°C, 50°C and 55°C resulted in a reduction of infectivity, although to different extents. By
188 contrast, Holder pasteurization (62.5°C for 30 min.) completely abolished PsV infectivity. Of note,
189 similar results were obtained when the Holder pasteurization was applied to PsV of an additional
190 high-risk type such as HPV-18 and to a low-risk type such as HPV-6 (Fig. 3). Moreover, the effect
191 of Holder pasteurization was not dependent on the PsV titer used as it was effective at HPV-16 PsV
192 concentrations up to 4 ng/ml of L1 capsid protein (Fig. 4). To rule out residual infectivity that
193 could not be revealed by the SEAP assay, GFP-expressing PsV were used as they allow the
194 detection of single infected cells by microscopy. As a control of the effectiveness of the Holder
195 procedure, the assay was run in parallel with a GFP-expressing HCMV, a virus which is known to

196 be completely inactivated by Holder pasteurization (11, 14). The results shown in Fig. 5
197 demonstrate that no GFP-expressing cells could be detected in cell monolayers infected with HPV-
198 16 PsV (panel A) or HCMV (panel B) that underwent Holder pasteurization. By contrast, a high
199 number of GFP-expressing cell are visible in monolayers infected with viruses maintained at 4°C
200 for 30 min as positive controls.

201 To further investigate the effect of the Holder pasteurization on HPV we analyzed the heat-treated
202 HPV-16 PsV by electron microscopy. Fig. 6 compares a sample subjected to Holder pasteurization
203 (panel B) to one maintained at 4°C for 30 min (panel A) as a control. As expected, in the control
204 panel several PsV particles are clearly visible. At a higher magnification (panel A, inset) these
205 particles appear as well assembled icosahedral capsids. By contrast, the analysis of the pasteurized
206 PsV revealed that the heat treatment completely destroyed the structural integrity of the viral
207 particles as no individual PsV can be observed (Fig. 6 panel B). Rarely, structures suggestive of
208 denatured, disassembled capsids can be detected at a higher magnification (panel B, inset).

209 Taken together these results demonstrates that HPV PsV are completely inactivated by Holder
210 pasteurization in a aqueous matrix such as a cell culture medium.

211

212 **Effect of Holder pasteurization on HPV PsV in human milk.** To determine the effectiveness of
213 Holder pasteurization on HPV in human milk, raw milk samples from donor mothers were spiked
214 with an inoculum of GFP expressing HPV-16 PsV or HCMV used a control. The inoculated milk
215 was then pasteurized at 62.5°C for 30 minutes in a water bath, a procedure currently in use in
216 human donor milk bank for milk pasteurization, or kept at 4°C. Diluted samples were then
217 inoculated into cell monolayers and examined for GFP expression. As shown in Fig. 7, no GFP-
218 expressing cells could be detected in cell monolayers infected with pasteurized HPV-16 PsV (panel
219 A) or HCMV (panel B). By contrast, a high number of GFP-expressing cells are visible in
220 monolayers infected with viruses maintained at 4°C for 30 min as positive controls. Similar results
221 were obtained when HPV-18 and HPV-6 PsV were used (data not shown).

222

223

224

225 **DISCUSSION**

226 The potential risk of infection in recipient infants raised by the recent detection of high-risk HPV in
227 human milk should be taken into account in the context of donor human milk banking. Currently,
228 donor selection and pasteurization of milk are the most important procedures that guarantee the
229 microbiological safety of human donor milk. Donor selection by serology or molecular diagnostic
230 techniques does not seem suitable to prevent a potential risk of HPV transmission through milk as
231 no concordance between cervical DNA detection and co-existent seropositivity was documented
232 (26). Moreover, no correlation was found between mothers' cervical or oral HPV results and HPV
233 detection in their breast milk (28, 22, 33). Besides, serology for HPV is not currently recommended
234 in donor mothers screening (1, 16, 24).

235 The Holder pasteurization is the most used and recommended method for the heat treatment of milk
236 in donor human milk banks as it allows a good compromise between the nutritional quality of milk
237 and its microbiological safety. Although its effectiveness to inactivate relevant viral pathogens
238 transmitted through milk, like HIV, HTLV-I, HTLV-II and HCMV, is well established, no data are
239 available for HPV. Therefore, the aim of this study was to determine whether the Holder method
240 completely inactivates HPV infectivity. We conducted our experiments both in cell culture medium
241 and human milk since other researchers have demonstrated the protective effects of milk on non
242 enveloped viruses such as hepatitis A virus (27, 4), poliovirus (30) and foot-and-mouth disease
243 virus (32). We did demonstrate that Holder pasteurization abolishes completely the infectivity of
244 high-risk HPV (i.e. HPV-16 and HPV-18) and low-risk HPV (i.e HPV-6) PsV in both matrices
245 indicating that milk fat and the casein proteins do not exert any protective effect on HPV PsV
246 particles.

247 It has been shown that virus inactivation by pasteurization depends on denaturation of viral
248 proteins, as well as disassembly of virus particles (29). To investigate the mechanism of HPV PsV
249 inactivation by Holder pasteurization we examined heat-treated PsV by electron microscopy.
250 According to what has been reported for other viruses (29) we observed that Holder pasteurization

251 induced HPV PsV particle disassembly which result in the generation of non infectious subunits.
252 Taken together, the results presented here indicate that the Holder pasteurization is an efficient
253 procedure to inactivate high-risk and low-risk HPV thus preventing the potential risk of their
254 transmission through human donor milk.

255

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261 continuous support to promote new studies with the aim to improve the quality of donor human
262 milk.

263

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364

365 **FIGURE LEGENDS**

366

367 Figure 1. Characterization of purified HPV-16 PsV. (A) An aliquot of purified PsV preparation was
368 analyzed by SDS-PAGE with Coomassie brilliant blue staining (lane 1) or immunoblotting with an
369 anti-L1 antibody (lane 2). (B) Electron micrograph of a purified PV preparation. Bar 100 nm.

370

371 Figure 2. Effect of different temperatures on HPV-16 PsV infectivity. PsV were incubated in cell
372 culture medium at 4°C, 25°C, 37°C, 45°C, 50°C, 55°C or 62.5°C for 30 minutes and then used to
373 infect 293TT cells for the measurement of SEAP expression. Results are given as the mean \pm
374 standard deviation of triplicates. The mean value was expressed as a percentage of the mean value
375 obtained at 4°C. * P value < 0.05 .

376

377 Figure 3. Effect of Holder pasteurization on HPV-18 and HPV-6 PsV infectivity. PsV were
378 incubated in cell culture medium at 4°C, or 62.5°C for 30 minutes and then used to infect 293TT
379 cells for the measurement of SEAP expression. Results are given as the mean \pm standard deviation
380 of triplicates. The mean value was expressed as a percentage of the mean value obtained at 4°C. P
381 value < 0.05 .

382

383 Figure 4. Effect of Holder pasteurization on increasing concentrations of HPV-16 PsV ranging from
384 1 to 4 ng/ml of L1 capsid protein. PsV were incubated in cell culture medium at 4°C, or 62.5°C for
385 30 minutes and then used to infect 293TT cells for the measurement of SEAP expression (CPS:
386 counts per second). Results are given as the mean \pm standard deviation of triplicates. The mean
387 value was expressed as a percentage of the mean value obtained at 4°C. P value < 0.05 .

388

389 Figure 5. Effect of Holder pasteurization on GFP expressing HPV-16 PsV (panel A) or HCMV
390 (panel B) in cell culture medium. PsV and HCMV were exposed to 4°C or to 62.5°C for 30 min and
391 then used to infect 293TT or HELF cells respectively. Cells were analyzed by confocal laser
392 scanning microscopy. The upper panels show the fluorescence images while the lower panels show
393 phase-contrast images.

394

395 Figure 6. Electron microscopy of purified HPV-16 PsV. (A) An aliquot of purified PsV preparation
396 was analyzed after incubation at 4°C for 30 min. Four distinct PsV particles are visible (indicated
397 by arrows). The inset shows a well assembled icosahedral capsid at a higher magnification. (B) An
398 aliquot of purified PsV preparation was analyzed after Holder pasteurization. No intact PsV are
399 visible. The inset shows a structure suggestive of a dysassembled capsid at a higher magnification.
400 Both images are at 120,000X magnification. This picture is representative of ten microscopic fields
401 examined for each experimental condition.

402

403 Figure 7. Effect of Holder pasteurization on GFP expressing HPV-16 PsV (panel A) or HCMV
404 (panel B) in human milk. PsV and HCMV were exposed to 4°C or to 62.5°C for 30 min and then
405 used to infect 293TT or HELF cells respectively. Cells were analyzed by confocal laser scanning
406 microscopy. The upper panels show the fluorescence images while the lower panels show phase-
407 contrast images.

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